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Cancer is a complex multistep disearse and progresses through successive accumulation of genetic mutations. Loss of tumor suppressor gene p53 and activation of oncogene Neu/ErbB2 are among the most frequent genetic alterations in human breast cancer. We performed a retroviral insertional mutagenesis screen to identify gene s that my contribute to mammary tumor formation in conjunctiong with deregulated p53 or Neu. Multiple proviral insertions form independent tumors were identified to be located within introns of the F-Box gene Fbw4, suggesting that the structural alteration at this locus may provide selective growth advantage. The viral integrations result in marked overexpression of a novel Fbw4 short isoform, which is also spontaneously enriched in several mouse and human breast cancer cell lines and thus appears to be associated with tumor formation. Overexpression of this short isoform in the 'normal' mouse mammary epithelial cell line NMuMg enable these cells to grow in soft agar. Taken together, these findings suggest that Fbw4 in a novel cancer-relevant gene. We propose a mechanism related to Fbw4's oncogenic properties.

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Introduction

Cancer is a complex multistep disease and progresses through accumulation and cooperation of genetic mutations. During tumor evolution, a number of the genetic alterations may make contributions to tumor progression and confer specific malignant features.

p53 is a tumor suppressor gene commonly altered in human cancers. p53 is capable of inducing growth arrest and apoptosis and loss of p53 activity gives an advantage to tumor growth (Vogelstein et al, 2000). Genetic studies in mice suggest that even a reduction in p53 gene dosage drives tumorigenesis (Venkatachalam et al, 1998). In many cases, p53 haplo-insufficiency collaborated with other cancer-causing events and significantly decreased tumor latency (Macleod and Jacks, 1999).

Neu/Her2/ErbB2 is an oncogene frequently amplified and overexpressed in human breast cancer (Hynes and Stern, 1994). Neu overexpression is an important stimulus to tumorigenesis. Transgenic mice expressing elevated levels of Neu in mammary epithelium produced mammary gland carcinomas with high efficiency (Hutchinson and Muller, 2000). However, the stochastic appearance of these tumors indicates that Neu by itself is not sufficient to fully transform mammary epithelial cells and additional genetic lesions are required for this phenotype.

Retroviral insertional mutagenesis using mouse mammary tumor virus (MMTV) has been a powerful mammalian genetic approach to discovering cancer genes (Callahan and Smith, 2000). MMTV infects and transforms mammary epithelial cells by insertional mutation of cellular proto-oncogenes or tumor suppressor genes. Mammary cells carrying such oncogenic insertions confer a growth advantage and preferentially grow out by clonal expansion to become a malignant tumor. Due to the essentially random viral integration into the host genome, common viral insertion sites are assumed to encode cancer-relevant genes (Mikkers et al, 2002). Indeed, many well-known oncogenes such as Wnt and Fgf have been discovered through this approach (Callahan and Smith, 2000).

Our goal is to identify new cancer genes involved in mammary tumor formation. In the present study we carried out a viral insertional mutagenesis screen by infecting p53 heterozygous and MMTV-Neu transgenic mice with C3H MMTV. We isolated and characterized genes that were targeted by MMTV integration in the arising mammary tumors. In addition to previously identified proto-oncogenes including Wnt and Fgf, we found several potentially interesting regulatory genes: the F-box and WD40-repeat gene Fbw4 (initially we called Fwd), the forkhead-domain transcription factor HFH1, and histone deacetylase gene HDAC6. Current study has been focused on Fbw4.

F-box proteins are generally a component of the so-called SCF (Skp1-Cullin-F-box protein) E3 ubiquitin ligase complex regulating ubiquitin-mediated protein degradation, which plays a fundamental role in determining the abundance of many critical regulatory proteins (Craig and Tyers, 1999). The ubiquitin-proteasome pathway requires the covalent attachment of polyubiquitin to substrate proteins. The ubiquitin transfer reactions involve the ubiquitin-activating enzyme E1, ubiquitin-conjugating enzyme E2 and ubiquitin ligase E3. Specificity of the ubiquitin-dependent proteolysis process is determined largely at the level of substrate recognition, a step mediated by E3. The substrate specificity of SCF E3 ligase complexes is determined by an F-box protein subunit. F-box proteins generally have a bipartite structure—the shared F-box motif, a modular domain linking F-box proteins to the core ubiquitination machinery via interaction with Skp1 (Bai et al, 1996), whereas divergent protein-protein interaction domains (e.g. WD40 repeats) selectively bind their cognate ubiquitination targets. We present evidence that Fbw4 may regulate protein abundance of p63, a p53-related factor.

Key Research Accomplishments (based on the Statement of Work)

Task 1: determine the transforming activity of the truncated F-box gene in cultured cells (months 1-12)

- We identified the Fbw4 gene as a common viral integration site

  Based on the sequence polymorphism of the C3H MMTV genome, we developed a high-throughput inverse PCR approach that allowed us to isolate C3H MMTV-specific viral integration sites. Viral insertions have been identified in introns of the Fbw4 locus from multiple independent tumors derived from both p53 heterozygous and MMTV-Neu transgenic mice. This result suggests that Fbw4 is a common viral integration site and MMTV-mediated structural alterations of Fbw4 may provide selective growth advantage and contribute to tumor formation.

- We identified a novel Fbw4 short isoform (Fbw4-s) induced by MMTV insertion

  MMTV retrovirus transforms host cells commonly through activation of cellular proto-oncogenes. In tumors harboring MMTV insertions at the Fbw4 locus, we detected marked overexpression of a novel short Fbw4 isoform (Fbw4-s), suggesting that Fbw4 is a candidate cancer gene. We further cloned the corresponding Fbw4-s cDNA encoding the short RNA species.

- Fbw4-s is spontaneously enriched in some mouse and human breast cancer cell lines

  By Northern blot analysis, we detected expression of Fbw4-s in a panel of mouse mammary tumor cell lines derived from MMTV-Neu transgenic mice, but not in 'normal' non-transformed mammary epithelial cells. Fbw4-s is also detected in a few human breast cancer cell lines. Therefore Fbw4-s is a naturally occurring isoform and appears to be commonly associated with both mouse and human mammary tumors.

- Overexpression of Fbw4-s confers oncogenic potential

  Transfection of Fbw4-s expression plasmids into rodent fibroblasts did not induce foci formation, a marker of cell transformation. However, stable clones of the immortalized mouse mammary epithelial cell line NMuMg overexpressing Fbw4-s acquired anchorage-independent growth ability in soft agar assay, suggesting that Fbw4-s confers transforming potential. These cells and Fbw4-s-expressing stable clones established in another mammary cell line EpH4 did not form tumors after injection into *nude* mice.

Task 2: generate and characterize transgenic mice overexpressing the truncated Fbw4 (months 6-36)

- Generating transgenic mice expressing a dominant-negative form of Fbw4 in mammary glands

  We initially reasoned that the short Fbw4 transcripts might encode a dominant-negative product. Therefore we designed an artificial dominant inhibitor of Fbw4: a construct retains all the WD40 repeats but lacks the putative F-box. The resultant peptide is expected to bind the potential Fbw4 substrates but fail to ubiquitinate them. We generated transgenic mouse lines that express this construct predominantly within the mammary glands under control of the MMTV long terminal repeat (LTR) promoter/enhancer. However, no mammary tumors have been detected so far in transgenic mice.

- Made transgenic construct for Fbw4-s

After determining the sequence identity of Fbw4-s, we made transgenic construct expressing Fbw4-s under regulation of MMTV LTR promoter/enhancer. Such construct may more closely mimic original viral insertion events and is expected to establish new transgenic mice.

Task 3: identify a protein substrate for the Fwd gene (months 1-18)

- Full-length Fbw4 is a component of SCF E3 ubiquitin ligase

By yeast two-hybrid and co-immunoprecipitation (co-IP) assays, we found that Fbw4 interacted with Skp1 via its putative F-box motif. This result suggests that Fbw4 is truly a component of E3 ubiquitin ligase involved in protein degradation control.

- p63 is a candidate substrate for the Fbw4 ligase complex

Fbw4 mutation in mice resulted in *dactylaplasia*, a limb defect phenotypically resembling typical human split hand/split foot malformation (SHFM) disease (Sidow et al, 1999). SHFM is genetically heterogeneous, and recently two SHFM loci have been identified as Fbw4 (de Mollerat et al, 2003) and p63 (Ianakiev et al, 2000; van Bokhoven et al, 2001), a p53 family member. These findings indicate that Fbw4 and p63 may act in the same genetic pathway. Moreover, p63 protein is degraded in part through a ubiquitin-proteasomal pathway (Ratovitski et al, 2001). Taken together, these observations raise the possibility that p63 might be a direct substrate for Fbw4.

We found that Fbw4 could indeed bind p63 in a co-IP assay, and enhance p63 ubiquitination. Depending on the usage of two promoters, p63 gene products can be divided into two types, TAp63 and ΔNp63 (Yang and McKeon, 2000). The predominant isoform expressed in vivo is ΔNp63α. In tumors harboring viral insertions in the Fbw4 locus, which likely impairs Fbw4 activity, ΔNp63α protein is accumulated. ΔNp63 isoforms lack the N-terminal transcription activation domain and act as oncogenic antagonists to p53 (Yang and McKeon , 2000; Hibi et al, 2000). Abnormally accumulated ΔNp63α is expected to interfere with p53 tumor suppressor function and contribute to tumor progression, providing a possible biochemical basis for collaboration between MMTV-deregulated Fbw4 and reduced p53 activities.

In summary, Task 1 has been completed, Tasks 2 and 3 are in progress.

Conclusion

We observed that a naturally occurring Fbw4 short isoform is dramatically induced by proviral insertions in mammary tumors. Such isoform is also enriched in some spontaneous mammary tumor cells and confers anchorage-independent growth capability, therefore possesses oncogenic properties. The full-length Fbw4 is a component of E3 ubiquitin ligase and may regulate p63 ubiquitination. We propose that viral integration impairs normal function of Fbw4 by disruption of one allele and induction of a short isoform. This leads to accumulation of downstream oncoproteins including ΔNp63 and contributes to tumor development.

Reportable Outcomes

Lu J and Leder P. Growth inhibition by the F-box factor Fbw4. Poster presented at the Cold Spring Harbor Laboratory meeting on "Cancer Genetics & Tumor Suppressor Genes", Cold Spring Harbor, New York. August 2002.

Lu J and Leder P. A potential role for the F-box gene Fbw4 in mammary tumorigenesis. Poster presented at the Salk Institute/EMBL meeting on "Oncogene & Growth Control", La Jolla, California. August 2003.

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